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Rapid Identification of Dengue Virus Serotypes by Using Polymerase Chain Reaction

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Four primer pairs were selected on the basis of the published sequence data of four dengue virus serotypes so that each unique target sequence size could be amplified for each serotype by polymerase chain reaction. The procedure consists of (i) RNA preparation, (ii) reverse transcription, and (iii) polymerase chain reaction, all of which could be completed within 2 h in a single tube for each specimen. The amplified sequence size revealed by ethidium bromide-stained agarose gel electrophoresis was unique for each serotype, using infected culture fluid of isolates from dengue fever or dengue hemorrhagic fever patients in Thailand, Indonesia, and the Philippines as well as from prototype viruses, thus facilitating simultaneous identification and typing.

Dengue viruses of four different serotypes (mosquito-borne flaviviruses) are causative agents of dengue fever and dengue hemorrhagic fever, which are highly prevalent in tropical countries, especially those of Southeast Asia (6). The identification and typing of dengue virus isolated from field-caught mosquitoes and from clinical specimens are important for epidemiological and clinical investigations. Development of type-specific dengue virus monoclonal antibodies has greatly facilitated these steps (8, 9), but it is still time-consuming. In this study, we developed a simple and rapid method for the detection, identification, and typing of dengue virus isolates by using polymerase chain reaction (PCR), a technique for DNA amplification in vitro (16, 18).

MATERIALS AND METHODS

Virus strains. The dengue virus strains used in this study are listed in Table 1 with their serotype, country of isolation, year of isolation, and clinical diagnosis. The viruses were inoculated once to C6/36 cells (10) in tube culture. Infected fluids were harvested 1 week after incubation at 28°C and stored at −80°C until use.

Primers. All primers were synthesized with an Applied Biosystems DNA synthesizer (model 380B) and confirmed for purity by ion-exchange gel chromatography (Gen-pack; Waters). When >1% of an incomplete length of oligomer was observed, the primer was discarded and the sequence was newly synthesized. The nucleotide sequence and reference information of the primers are listed in Table 2.

RT-PCR. (i) Rapid RT-PCR. Five microliters of infected fluid was incubated with an equal volume of detergent mix (1% Nonidet P-40, 10 U of RNase inhibitor [Takara Co., Kyoto, Japan] in phosphate-buffered saline [−]) in a 500-μl Eppendorf-type tube for 1 min at room temperature. This was followed by the addition of 90 μl of reverse transcription (RT)-PCR mix (100 pmol of each primer, 0.2 mM deoxynucleoside triphosphate, 10 mM Tris [pH 8.9], 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 0.1% sodium cholate, 0.1% Triton X-100, 10 U of reverse transcriptase [Life Science Inc.] and 2 U of Tth DNA polymerase, a thermostable DNA polymerase [Toyobo Co., Osaka, Japan]). The reaction mixture was covered by 2 drops of mineral oil, and the tube was incubated for 10 min at 53°C for RT. PCR amplification (92°C for 60 s, 53°C for 60 s, and 72°C for 60 s by thermal cycler; Iwaki Co., Tokyo, Japan) was started immediately after the RT and repeated 25 to 35 times. Five microliters of PCR product was subjected to agarose gel electrophoresis, and amplified DNA fragments were visualized by ethidium bromide staining.

(ii) Standard RT-PCR used in Fig. 1. Five microliters of RNA template was heated at 95°C for 3 min and added with 5 μl of complementary primer (100 pmol) in a 500-μl Eppendorf-type tube. The mixture was cooled down to 42°C and incubated for 5 min at that temperature. This was followed by the addition of 40 μl of RT mix (0.2 mM deoxynucleoside triphosphate, 10 mM Tris [pH 8.9], 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 10 U of reverse transcriptase). The mixture was incubated at 53°C for 10 min for RT followed by the addition of 50 μl of the following mixture: 100 pmol of sense primer, 0.2 mM deoxynucleoside triphosphate, 10 mM Tris [pH 8.9], 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 0.2% sodium cholate, 0.2% Triton X-100, and 2 U of Tth DNA polymerase. The reaction mixture was covered by 2 drops of mineral oil, and the PCR incubation was performed as described for the rapid RT-PCR procedure.

Dot blot hybridization. Ten microliters of each PCR product was heat denatured at 100°C for 5 min and mixed with 10 μl of 2 M ammonium acetate. The mixture was blotted on a Hybond N membrane (Amersham), using a Bio-Dot apparatus (Bio-Rad). The membrane was washed once with 1 M ammonium acetate, soaked in alkali solution (0.5 M NaOH, 1.5 M NaCl) for 10 min, and neutralized in 0.5 M Tris HCl (pH 7.5)–1.5 M NaCl solution for 5 min. The membrane was dried and irradiated by UV light (256 nm) for 3 min.

The membrane was prehybridized with hybridization buffer containing 45% formamide (supplied by Amersham) at 42°C for 1 h. Molecularly cloned cDNA (50 ng) for each dengue virus serotype, containing the target nucleotide sequences, was labeled with ^{32}P-dCTP by using a nick translation labeling kit (Takara Co.) in accordance with the manufacturer's protocol. The radiolabeled probe was added to the prehybridization buffer, and the membrane was incubated overnight at 42°C.

After the hybridization, the membrane was washed with 1 × SSC (150 mM NaCl and 15 mM sodium citrate)–0.1%

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TABLE 1. Information on dengue virus strains

Strain	Type	Country	Yr	Diagnosis[a]
D1 (Hawaiian)	D1	Prototype		
D2 (New Guinea B)	D2	Prototype		
D80-709	D2	Thailand		
PhMH10-84	D2	Philippines	1984	
D80-753	D2	Thailand	1980	
D3 (H87)	D3	Prototype		
U20-82	D3	Indonesia	1982	FUO
I6-82	D3	Indonesia	1982	DHF
ThCMP15-82	D3	Thailand	1982	DHF
16562	D3	Philippines	1964	DHF
PhMH2311-83	D3	Philippines	1983	
PhMH4-84	D3	Philippines	1984	
V11	D3	Indonesia	1976	DF
D4 (H241)	D4	Prototype		
D80-785	D4	Thailand		
SI-YO SMB10	D4	Thailand	1978	DHF
No. 17	D4	Sri Lanka	1978	

[a] FUO, fever of unknown origin; DHF, dengue hemorrhagic fever; DF, dengue fever.

sodium dodecyl sulfate for 10 min at room temperature once and at 42°C for 15 min three times. Then the membrane was dried and exposed to Amersham hyperfilm with intensifying screen at −80°C overnight.

RESULTS

Simplification of the RT-PCR procedure. To simplify the RT-PCR procedure, four different procedures were compared (Fig. 1). The lane 1 method is a standard RT-PCR procedure in which RNA is purified by phenol-chloroform extraction and heat denatured, followed by annealing of complementary primer (D2C) and RT prior to the PCR cycle. This procedure involves at least two handlings and incubations of tubes in a water bath prior to the PCR cycle. In lane 2, detergent-treated infected fluid is used as template instead of the purified RNA used in the lane 1 procedure. Lane 4, or rapid RT-PCR, was performed as described in Materials and Methods, using 5 μl of infected fluid (5 × 10^5 PFU/ml). The results of rapid RT-PCR, using purified RNA as template, are displayed in lane 3.

An amplified target DNA band of rapid RT-PCR with purified RNA as template (lane 3) showed a density as good as that in the standard RT-PCR procedure (lane 1). Furthermore, infected fluid treated by detergent was just as good a template as purified RNA in rapid RT-PCR (lane 3 and 4). All procedures could be completed within 2 to 3 h.

Sensitivity of rapid RT-PCR (Fig. 2). Infected fluid of dengue virus type 2 was diluted 10-fold with Eagle minimum essential medium, and 5 μl of each diluted sample was subjected to the rapid RT-PCR procedure, using primers D2S and D2C to evaluate the detection limit. After 25 cycles, the amplified target DNA band was detected in 1:100-diluted samples, or approximately 25 PFU of virus in each reaction tube (Fig. 2a). By 35-cycle amplification, positive results were obtained from 1:1,000-diluted samples, corresponding to 2.5 PFU/reaction tube (Fig. 2b). Thus, the sensitivity of rapid RT-PCR, using infected fluid, is sufficient to detect dengue virus genome in infected fluids of 500-PFU/ml virus titer.

Serotype specificity of each primer pair. The sequences of

TABLE 2. Nucleotide sequences of dengue virus primers

Code[a]	Sequence	Position	Reference(s)
D1S	GGACTGCGTATGGAGTTTTG	2229–2248	15
D1C	ATGGGTTGTGGCCTAATCAT	2718–2699	15
D2S	GTTCCTCTGCAAACACTCCA	1203–1222	3, 4
D2C	GTGTTATTTTGATTTCCTTG	1432–1413	3, 4
D3S	GTGCTTACACAGCCCTATTT	2253–2272	17
D3C	TCCATTCTCCCAAGCGCCTG	2572–2553	17
D4S	CCATTATGGCTGTGTTGTTT	3973–3992	14, 20
D4C	CTTCATCCTGCTTCACTTCT	4370–4351	14, 20

[a] S, sense primer; C, complementary primer; D1, -2, -3, -4; dengue virus types 1, 2, 3, and 4.

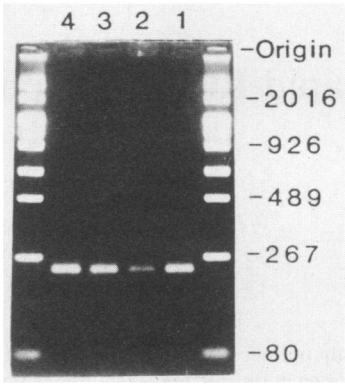

FIG. 1. Photograph of an agarose gel stained with ethidium bromide, containing PCR products obtained by four different procedures and using the dengue virus type 2 primer pair (D2S and D2C). (Lane 1) Purified RNA prepared from 5 μl of infected fluid (5 × 10^5/ml) was heat denatured and annealed with complementary primer (D2C) at 42°C for 5 min and reverse transcribed for 10 min at 53°C, as described in Materials and Methods. After RT, PCR mix containing sense primer and Tth DNA polymerase were added (final volume, 100 μl) and amplified. (Lane 2) The same volume of infected fluid treated with Nonidet P-40 and RNase inhibitor, as described in Materials and Methods, was used as template and treated as in lane 1. (Lane 3) Purified RNA was used instead of infected fluid. The rest of the procedure is the same as described for lane 4. (Lane 4) Rapid RT-PCR was performed as described in Materials and Methods. All PCR amplifications were done 25 times.

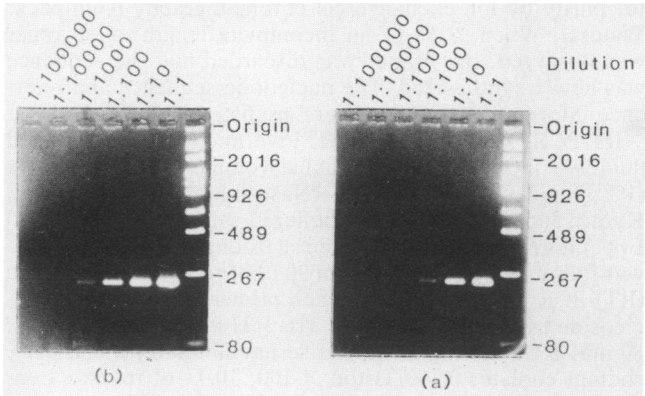

FIG. 2. Sensitivity of rapid RT-PCR for detection of dengue virus type 2. Infected fluid was serially diluted in 10-fold steps with minimum essential medium containing 10% fetal calf serum and subjected to rapid RT-PCR as described in Materials and Methods. The virus amount (1:1) was about 2.5 × 10^3 PFU in one reaction tube. (a) Amplified 25 PCR cycles; (b) amplified 35 PCR cycles.

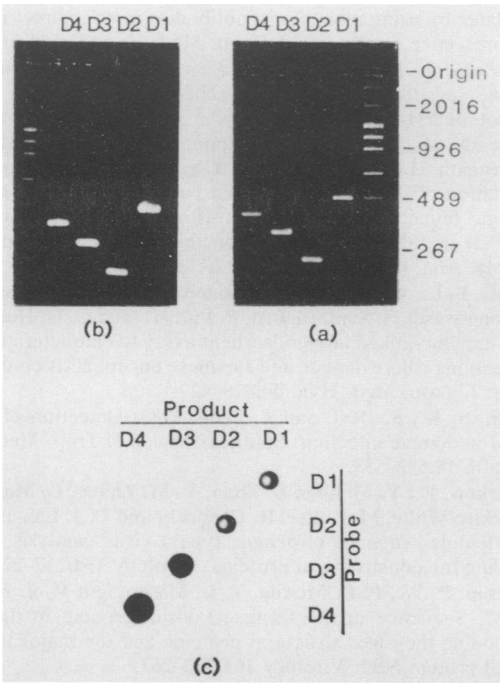

FIG. 3. Identification of dengue virus serotype by dengue virus-specific primer pairs. Infected fluid (5×10^5 to 8×10^7 PFU/ml) of each dengue prototype virus was mixed with detergent mix containing (a) only homologous primer pairs (100 pmol each) or (b) four sets of primers (D1S and D1C, D2S and D2C, D3S and D3C, and D4S and D4C, 100 pmol each) and subjected to the RT-PCR procedure as described in Materials and Methods. D1, dengue virus type 1; D2, dengue virus type 2; D3, dengue virus type 3; D4, dengue virus type 4 prototype viruses. Dengue virus types 1, 2, 3, and 4 show unique lengths of amplified DNA fragment: 490, 230, 320, and 398 bp, respectively. (c) Dot blot hybridization with cloned cDNA probe of each dengue virus serotype. The PCR product of each dengue virus type in panel b was dotted on the membrane and hybridized with each serotype-specific radiolabeled probe, as described in Materials and Methods.

dengue virus serotype-specific primer pairs were selected from published data (3, 4, 14, 15, 17, 20) by computer analysis. Each primer pair reacted well to its homologous virus, and the size of each amplified DNA corresponded closely to the expected size (490, 230, 320, and 398bp for D1, D2, D3, and D4, respectively), which was calculated from the nucleotide sequences (Fig. 3a). The sensitivities of D1, D3, and D4 primer pairs are 8, 3.0, and 3.5 PFU/reaction tube, respectively.

All four primer pairs were then mixed and examined to determine whether or not heterologous primers create a background DNA band in addition to the target DNA band. Figure 3b shows that only the target DNA fragments were amplified even when four sets of primers were present in the RT-PCR reaction mixture, indicating a high specificity of each primer pair for its homologous serotype.

We investigated the cross-reactivity of these four primer pairs to other flaviviruses. Dengue type 1, Japanese encephalitis, St. Louis encephalitis, Murray Valley encephalitis, and West Nile viruses were examined, using $>10^5$ PFU of each virus per tube in the reaction mixtures. No obvious DNA band was observed on other flaviviruses after 35 PCR cycles (data not shown), but the RT-PCR with flavivirus cross-reacting sequence primers showed significant bands for the flaviviruses (data not shown).

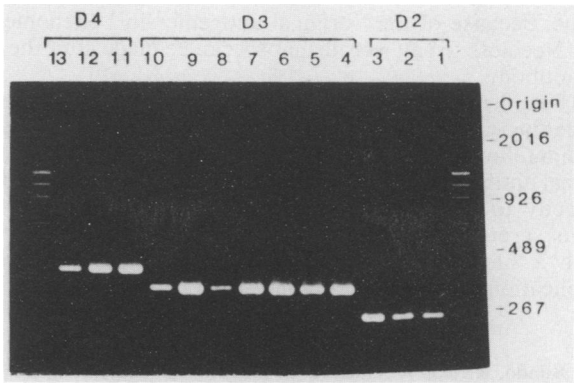

FIG. 4. Photograph of an agarose gel stained with ethidium bromide, demonstrating the serotype specificity of the four primer pairs among Southeast Asian dengue virus strains. The results are in complete agreement with serological results. Lanes; 1, strain D80-709; 2, PhMH10-84; 3, D80-753; 4, U20-82; 5, I6-82; 6, ThCMP15-82; 7, 16562; 8, PhMH2311-83; 9, PhMH4-84; 10, V11; 11, D80-785; 12, SI-YO SMB10; 13, No. 17.

These observations demonstrate the specificity of the primer pairs for each dengue virus serotype among the flaviviruses.

Reactivity of the primers to various isolates in Southeast Asia. We investigated the specificity of the primer pairs to dengue virus strains isolated from patients' sera in Southeast Asia. Figure 4 clearly shows the serotype specificity of the four primer pairs for the dengue type 2, 3, and 4 virus strains in Southeast Asia examined thus far.

DISCUSSION

The four sets of primers we selected each showed type-specific reactivity with sufficient sensitivity to the isolates collected in Thailand, Indonesia, and the Philippines as well as to prototype viruses, indicating that the sequences of each primer were well conserved in each dengue virus serotype isolate in Southeast Asia and highly specific to the respective serotype.

The C6/36 cells (10) can be used for dengue virus isolation from patients' sera and from field-caught mosquito homogenates as an improved virus isolation method (11, 19). We examined C6/36 cell culture fluids inoculated with patients' sera by using our rapid RT-PCR method and confirmed that the system worked very well on these primary infected cell culture fluids (data not shown). Since all procedures of rapid RT-PCR were completed within 2 h and the results of the serotypes were consistent with those determined by immunological examination (immunoperoxidase staining of the infected cells by using dengue virus type-specific monoclonal antibodies), in which 16 samples were in agreement (unpublished data), the method was shown to be useful for clinical and field specimens.

Since the number of cases of dengue virus infection is increasing (6), the disease is a serious health problem in tropical areas. It is important, therefore, to have a reliable method to prove the virus infection and to identify the serotype for clinical diagnosis and epidemiological study. Laboratory diagnosis of primary dengue virus infection can be performed by immunoglobulin M-capture enzyme-linked immunosorbent assay (1, 2, 5, 12, 13) after seroconversion. However, it is rather difficult to identify the serotype of the infecting virus, particularly in secondary infection of dengue

virus, because of the "original antigenic sin" phenomenon (7). Needless to say, serodiagnosis can be made after the rise in antibody.

Thus, the most conventional and reliable method to determine the virus serotype is virus isolation from the patient's serum followed by immunological examination, using monoclonal antibodies (8, 9). If the method we describe is applied directly to dengue virus patients' sera, the diagnosis and virus serotype identification can be done at the same time with a single-tube reaction. We are planning the clinical application of this method in an epidemic area.

REFERENCES

1. **Bundo, K., and A. Igarashi.** 1985. Antibody-capture ELISA for detection of immunoglobulin M antibodies in sera from Japanese encephalitis and dengue hemorrhagic fever patients. J. Virol. Methods **11**:15–22.
2. **Burke, D. S.** 1983. Rapid methods in the laboratory diagnosis of dengue virus infections, p. 72–84. *In* T. Pang and R. Pathmanathan (ed.), Proceedings of the International Conference on Dengue/Dengue Hemorrhagic Fever. University of Malaya, Kuala Lumpur, Malaysia.
3. **Deubel, V., R. M. Kinney, and D. W. Trent.** 1986. Nucleotide sequence and deduced amino acid sequence of the structural proteins of Dengue type 2 virus: Jamaica genotype. Virology **155**:365–377.
4. **Deubel, V., R. M. Kinney, and D. W. Trent.** 1988. Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of Dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. Virology **165**:234–244.
5. **Gunasegaran K., T. W. Lim, A. Ahmed, J. G. Aaskov, S. K. Lam, and T. Pang.** 1986. Hemadsorption immunosorbent technique for the detection of dengue immunoglobulin M antibody. J. Clin. Microbiol. **23**:170–174.
6. **Halstead, S. B.** 1980. Dengue haemorrhagic fever—a public health problem and a field for research. Bull. W.H.O. **58**:1–21.
7. **Halstead, S. B.** 1988. Pathogenesis of Dengue: challenges to molecular biology. Science **239**:476–4818.
8. **Henchal, E. A., M. K. Gentry, J. M. McCown, and W. E. Brandt.** 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. **31**:830–836.
9. **Henchal, E. A., J. M. McCown, M. C. Seguin, M. K. Gentry, and W. E. Brandt.** 1983. Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. Am. J. Trop. Med. Hyg. **32**:164–169.
10. **Igarashi, A.** 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol. **40**:531–544.
11. **Igarashi, A., N. Fujita, Y. Okuno, T. Oda, Y. Funahara, A. Shirahata, H. Ikeuchi, S. Hotta, A. S. Wiharta, and Sujudi.** 1982. Isolation of dengue viruses from patients with dengue hemorrhagic fever (DHF) and those with fever of unknown origin (FUO) in Jakarta, Indonesia, in the years of 1981 and 1982. ICMR Ann. (Kobe University) **2**:7–17.
12. **Innis, B. L., A. Nisalak, S. Nimmannitya, S. Kusalerdchariya, V. Chongswasdi, S. Suntayakorn, P. Puttisri, and C. H. Hoke.** 1989. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am. J. Trop. Med. Hyg. **40**:418–427.
13. **Lam, S. K., S. Devi, and T. Pang.** 1987. Detection of specific IgM in dengue infection. Southeast Asian J. Trop. Med. Public Health **18**:532–538.
14. **Mackow, E., Y. Mokino, B. Zhao, Y. M. Zhang, L. Markoff, A. Buckler-White, M. Guiler, R. Chanock, and C. J. Lai.** 1987. The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. Virology **159**:217–228.
15. **Mason, P. W., P. C. McAda, T. L. Mason, and M. J. Fournier.** 1987. Sequence of the Dengue-1 virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology **161**:262–267.
16. **Mullis, K. B., and F. A. Faloona.** 1987. Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. Methods Enzymol. **155**:335–350.
17. **Osatomi, K., and H. Sumiyoshi.** 1990. Complete nucleotide sequence of Dengue type 3 virus genome RNA. Virology **176**:643–647.
18. **Saiki, R., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
19. **Tesh, R. B.** 1979. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am. J. Trop. Med. Hyg. **28**:1053–1059.
20. **Zhao, B., E. Mackow, A. Buckler-White, L. Markoff, R. M. Chanock, C. J. Lai, and Y. Makino.** 1986. Cloning full-length dengue type 4 viral RNA sequence: analysis of genes coding for structural proteins. Virology **155**:77–88.